THE FLUORESCENT ANTIBODY METHOD OF DIAGNOSING ANTHRAX

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[Following is a translation of an article by Dr. E.H. Kampelmacher of the Netherlands Public Health Laboratory for Zooenoses in the Dutch-language periodical Tijdschrift voor Diergeneeskunde (Veterinary Journal) 85: 1802-1813, 1960.]

Ladies and Gentlemen.

The information day of the Veterinary Service, which among other things, attempts this year to throw light on the problem of anthrax diagnosis, gives me the opportunity to inform the Dutch veterinarean of a new diagnostic method of which he probably will hear more and more in the future. However, the method is not quite new because Landsteiner speaks already in his fundamental work Die Spezifitaet der serolcgischen Reaktionen of fluorescent antibodies (FA). In 1941, Coons started his research on this method but, mainly because of the war, this work was delayed. In 1950 Coons and his collaborators published the results of their experiments upon which the practical application of the FA method is based. The US Federal Civil Defense Administration immediately recognized the great possibilities of this method for a quick identification of biological means of warfare and strongly stimulated the researchwork financially. Numerous publications of the last years testify to the intensity of the research and excellent results accomplished, mainly in the United States. In the first place should be mentioned a research team studying since 1952 at the Communicable Disease Center in Atlanta the practical use of this method in cases of parasitological bacterial and virus diseases. Goldman (1953) informed of the successful application of marked antibodies in diagnosing Entamoeba histolytica, which is of the greatest importance for the differentiation from the closely but non-pathogen Entamoeba coli. In 1957 the same investigator succeeded by means of the FA method in showing Toxoplasma goudii in smears of tissue material. Thomason, Moody and Goldman (1956) described the possibilities of diagnosing with the FA method Malleomyces pseudomallei in contaminated material of infected animals. Thomason, Cherry and Edwards (1959) published their results of the identification of Salmonellae in feces samples. Thomason, Cherry and Ewing (1959) investigated the possibility of a quick diagnostic method of pathogen E. coli serotypes. Smith and Marshall succeeded in distinguishing L. Moncytogenes by

means of FA, and Cherry and Freeman (1959) used this method in anthrax diagnosis.

This is only a few of the very many publications of the last years. At the same time it was my intention to make you conversant with the names of some investigators who have done good work in elaborating this diagnostic method. In 1959 I had the privilege of observing their work.

Apart from this, research on the FA method has not been limited to the United States. In Great Britain it is mainly Chadwick, in the Soviet Union Mikhailov and Levina and in Austria Petuely and Lindner who have experimented in the field of the FA method. Regularly, publications appear in which the value of this method in tracing parasites, bacteria and virusses is described, and there are at this moment few pathogen micro-organisms that have not been a part of the FA research. That also in our country this diagnostic method comes more and more in the center of attention is proven by the symposium that within a few days will take place in Leiden and will be devoted to the technique and the practical use of the FA method.

After this short historical introduction the time has come to tell you something about the method itself. The method makes it possible to bring into direct view, by means of a smear under a microscope, an antigen-antibody combination, classically only visible in agglutination, precipitation or complement-attaching reactions. To achieve this the globulin fraction of the antibodies, that is, of anti-serum obtained, for instance, from a rabbit after injection of a certain antigen, is brought together with strong color retaining material. This bringing-together is called labeling, and strongly fluorescent substances as, for instance, isothiacyanate, isocyanate and rodamin are used for the purpose. If the antigen (bacteria, virusses(is now brought together with the labeled globulin fraction of the homologue antibodies, then on the surface of the antigen parts, for instance bacteria, an attaching between antibodies and antigen will take place. This attaching can be made visible by means of a dark field fluorescence microscope. In a thin smear, for instance, of separate lying bacteria a strong light-giving coat becomes visible. Depending on the preparation and the optical apparatus, the colored light, called the staining, is bright green (cyanates) or light pink (rodamin). This phenomenon only occurs when the antigen is brought together with its homologue labeled antibody, so it is very specific. Because there is here a direct antigen-antibody reaction and no macroscopic particles have first to appear, as in the classical methods, the FA method is not bothered by prozone effects.

Without mentioning the many problems of the preparation of coupled globulin fractions, the choice of the optical apparatus and the fluorescence, I would like to explain to you the many possibilities of this method briefly. The simplest way is the direct test in which a labeled known antiserum, for instance

rabbit serum against B. anthracis is brought together with a suspect but unknown antigen, B. anthracis, for instance, in a smear on a slide. If "the key fits the lock," that is if this labeled anti-anthrax serum is indeed brought together with anthrax bacilli, then the bacilli with their strongly lighted coat will be visible under the fluorescence microscope (Figure I). If the bacilli are not B. anthracis, no light will be visible.

A second method is the so-called inhibition test. In this case a known antigen is brought together with an unknown, unlabeled serum on a slide, B. anthracis-germs, for instance, with serum of bovine cattle suspected of anthrax. If this combination is now brought together with a labeled B. anthracis antiserum, then no attaching takes place and no light will be visible under the fluorescence microscope. This negative result is proof of a positive diagnosis. (Fig. II).

It goes without saying that in this test a possitive check is always made at the same time with a known positive anti-anthrax serum and a negative normal serum. With this inhibition method it is also possible by dilution of the used unlabeled antiserum to determine rather accurately the titer of the antibodies.

A third and last method is the indirect test. In this case a known unlabeled serum, anti-anthrax rabbit serum, for instance, is brought together with an unknown unlabeled antigen, B. anthracis, for instance. If this unlabeled product is brought together with a labeled anti-rabbit serum, prepared in a sheep, then a specific attachment will be clearly visible under the fluorescence microscope. In this way the unlabeled anti-serum plays a double part: as an antiserum in the first phase of the reaction, and as an antigen in the second phase. With the indirect test an unknown serum as well as an unknown antigen can be diagnosed. With this method, also, it is possible by dilution to determine the titer of the unlabled serum. The great advantage of the indirect test is that it is only necessary to label one antiglobin (in practice most times anti-rabbit) that can be used as an indicator in different combinations between antigen and unlabeled antiserum. (Figure III).

It is clear that the FA method is of great importance as long as we do not have other quick and trustworthy diagnostic methods at our disposal. That this is the case in B. anthracis will be known to many of you who are confronted with the necessity of a quick anthrax diagnosis, and it has been confirmed by this lecture.

On this basis we have attempted for some time, in collaboration with Cherry and Freeman, who in 1959 published their results on the diagnostic value of labeled anti-anthrax sera, to obtain sera making a quick and trustworthy diagnosis of B. anthrax possible. It can't be denied that the work as to the production of serum preparation, of labeling and coupling, still involves many difficulties. To improve the specifity -- the different genera of the Bacillacea family show a strong mutual relationship --

B. anthracis coat-antigen-antiserum is prepared. Then the globulin fraction of this serum is coupled with isothiocyanate and absorbed to mouseliver powder to neutralize unspecific reactions. Notwithstanding this, B. anthracis, closely related to B. cereus, also shows light if this germ is brought together with labeled anti-anthrax serum. This bringing-together is done on a cover glass on which a thin smear of a blood or tissue sample is made and to which a drop of the labeled serum is added. To the trained investigator the light of B. anthracis is different from that of B. cereus, but the method is not yet suitable for general diagnostic use. It is possible that further research will raise the specifity for B. anthracis sufficiently to make a sure and quick diagnosis by means of the FA method possible. This is the reason that at this moment I can only show you a picture of the future: labeled, specific anti-anthrax sera centrally prepared, with which you can make a fairly sure diagnosis in the nearest laboratory possessing a fluorescence microscope. The realization of this prospect is the subject of research in several laboratories.

SUMMARY.

After a historical survey regarding the diagnostic method by means of fluorescent antibodies (FA) the various possibilities in this test are further discussed, and the value of the method for anthrax diagnosis is examined. The difficulties that still exist in the practical application of the method are pointed out.

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DISCUSSION following the lectures on anthrax.

Question:

Prof. Dr. P. Muntendam expressed his thanks to Mr. van den Born for the invitation to be present at this informative meeting. He appreciated the choice of the subject -- anthrax --, because anthrax is also of great importance in human medicine. The speaker had investigated by means of the files of the Rijksverzekeringsbank (Government Social Insurance Institute) the frequency in 1939-1941 of anthrax in industrial, farm and agricultural personnel. During those years the number of anthrax cases did not increase. He would like to know whether the information and education of farmers resulted in the decrease in this category in comparison to the category of those employed in industries working with hides and other material of animals that died of anthrax. The conclusion could be that contamination is not primarily caused by the cattle farm business.

Answer:

Mr. Karsemeijer replied that indeed anthrax is more prevalent in persons working in the hide, wool, and hair industry than in farmers and their personnel. When during the epizooety of 1940 the cattle raisers were informed and warned, they were all the time very watchful, and that is why the "Rijksverzekeringsbank" then did not get more reports of anthrax from the cattle people. However, when there are, during a certain period of time, only a few anthrax cases, the attention and carefulness of the farmer weakens and cases of anthrax in human beings appear again.

Question:

In the Netherlands East Indies Prof. A. v.d. Schaaf often experienced difficulty in diagnosing anthrax in not fresh cadavers sent to him. Research was done by Prof. Dr. Kraneveld and his collaborators about the time after death in which it was still possible to show anthrax from material of closed cadavers. He asked whether it is known in the Netherlands how long after death of an animal not previously treated with antibiotics it is possible to show B. anthracis. Prof. v.d.S. mentioned the method used in the East Indies, namely to dip a wetted bar of plaster in the blood of the cadaver and let it dry in the sun before sending it to the laboratory. Here the outer layer is scraped off, this material is mixed with physiological NaCl-solution, heated to 70°C and after this, growing tests and cavia tests were done. This way a much longer time is available for diagnosing because of the possibility of spores being produced.

Answer:

Dr. Hill replied that the material he receives is in most cases an ear or other material of a cadaver, but often he is not informed about the time of death. If we find anthrax in this material our diagnosis is positive. If we don't find anthrax we make the statement: no anthrax. This does not mean that there was actually no anthrax in the body of the animal, but that with the method used we were not able to find it. It is difficult to say for how long anthrax can be detected in a closed cadaver. With the material we obtain we have too little experience.

Remark:

Dr. J. M. van Vloten remarked that all slaughter animals, also when they die or are killed in case of necessity, are subject to inspection and examination according to sections 4 and 5 of the Meat Inspection Law. Section 29 of this Law states that animals that are not to be butchered can only be declared unfit for use after anthrax is excluded. Here lies the responsibility of the Veterinary Inspector. Section 30 of the Inspection Regulations orders an anthrax investigation of animals that have died and are to be butchered.

Commentary:

Mr. Karsemeijer stressed the point that only the Veterinary Inspector is responsible for no anthrax case escaping his attention. In a great number of cases he must make an anthrax test, but this may be omitted in case of parts of a fetus after embryotomy, very young pigs, and of other material to be destroyed.

Question:

Dr. H. H. Scholten remarked that in the lecture of Mr. H. Tj. v.d. Veen it is stated that the milk of feverish animals should be declared unfit for use. Is it true that anthrax bacilli already appear in the milk as soon as the animal is ill, or do they appear only in the agony stage?

Answer:

Mr. Kraai stated that anthrax bacilli can be expected in the milk of a feverish animal. It will not be difficult to disinfect contaminated milk with creoline to avoid further contamination. In case of mastitis in a dairy, the possibility has to be considered that this mastitis may be caused by anthrax as a second instance, possibly even as a first instance.

Remark:

Dr. K. Reitsma: I call your attention to the simplest diagnostic method not yet mentioned here this morning, namely the native preparation. The speaker regularly made use of this method in cases of dead animals on suspected farms and pastures during an orienting investigation. In his auto he had a microscope, some object- and cover glasses and a small knife. A little cut in the lower ear, a drop of blood, and a local microscopic examination was made. Many bars floating among the red (yellow) blood corpuscles prove with practical certainty the presence of anthrax. After that the laboratory examination was made and the Veterinary Inspector was informed. If no bacilli are found, anthrax can practically be excluded and the animal may be transported for inspection and slaughtering.

Question:

W. P. A. Colsen asked if there is any objection to using the penicillin-agar method for routine examination. He remarked further that anthrax is often taken for Clostridium infections, which also give rise to invaginations. He observed never having seen anthrax in deductor personnel, although cadavers are often carelessly handled. Would there still be another necessary factor to cause anthrax?

Answer:

Dr. Kampelmacher replied to Dr. Reitsma's remark. The problem has two facets: the laboratory and practice. In practice, one is not supposed to take risks and needs a wider scope than in the laboratory. On the other hand, in the laboratory one wishes to know

with certainty what is being handled. The microscope in the car may save you mistakes, but scientifically it is not satisfactory. In this way we don't get an ultimate idea of the occurrence of anthrax in the Netherlands. To answer the question of Mr. Colsen, Dr. Kampelmacher did not think that there is any objection to use of the penicillin bead string test. However, more than one method should be used. In the laboratory three to four anthrax tests are practised at the same time. One should be careful not to consider the result of one method as an absolute certainty, the complexity of the problem is too great.

The question of anthrax-infected cadavers and the personnel of destruction enterprises can be briefly answered: in daily study of diseases and germs it is most remarkable to observe the tremendous power of natural immunity.

Mr. Karsemeijer next noted the description of an anthrax case of an ox with clearly shown invagination. Referring to anthrax bacilli in milk, the speaker said that it happens that in a stable with anthrax cases a case of mastitis is once in a while to be seen. Extraordinary care is necessary here, because this could be caused by anthrax. It happens also that when such a mastitis is healed, anthrax bacilli can be found in the secretion, even after a month. In an Austrian publication cases of mastitis are also described, caused by anthrax bacilli; the bacilli were found in the milk. The speaker finally called attention to the fact that there are still unrecognized cases of anthrax, because no thought was given to the possibility. That is why destructors are still supplied with anthrax cadavers.

Illustration Captions.

Fig. I

Diagram of the Direct Coloring

a) Coupled antibody b) Non-coupled antibody c) Coupled product

Fig. II

Diagram of the Inhibition Test

a) Non-coupled antibody b) Antigen c) Non-coupled product

d) Coupled antibody e) Non-coupled product f) Non-coupled product

Fig. III

Diagram of the Indirect Coloring

a) Non-coupled antibody (rabbit)

b) Non-coupled antigen

c) Non-coupled product

d) Coupled antibody (anti rabbit-sheep)

e) Non-coupled product

f) Coupled product